Effects of aerobic interval training and continuous training on cellular markers of endothelial integrity in coronary artery disease: A SAINTEX-CAD substudy.

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Running title: Effect of exercise training on EPC, angiogenic T cells and EMP in CAD

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ABSTRACT

Background: In this large multicenter trial, we aimed to assess the effect of aerobic exercise training in stable coronary artery disease (CAD) patients on cellular markers of endothelial integrity, and examine their relation with improvement of endothelial function.

Methods: Two-hundred CAD patients (LVEF >40%, 90% male, mean age 58.4±9.1 years) were randomized on a 1:1 base to a supervised 12-week rehabilitation program of either aerobic interval training (AIT) or aerobic continuous training (ACT) on a bicycle. At baseline and after 12 weeks, numbers of circulating CD34+/KDR+/CD45dim endothelial progenitor cells (EPC), CD31+/CD3+/CD184+ angiogenic T-cells and CD31+/CD42b- endothelial microparticles (EMP) were analyzed by flow cytometry. Endothelial function was assessed by flow-mediated dilation (FMD) of the brachial artery.

Results: After 12 weeks of AIT or ACT, numbers of circulating EPC, angiogenic T-cells and EMP were comparable to baseline levels. Whereas improvement in peak VO_2 was correlated to improvement in FMD (pearson r = 0.17, p = 0.035), a direct correlation of baseline or post-training EPC, angiogenic T-cells and EMP levels with FMD was absent. Baseline EMP related inversely to the magnitude of the increases in peak VO_2 (spearman rho = -0.245, p = 0.027) and FMD (spearman rho = -0.374, p = 0.001) following exercise training.

Conclusions. Endothelial function improvement in response to exercise training in CAD patients is not mediated by increased releases of EPC and angiogenic T-cells and/or a diminished shedding of EMP into the circulation. EMP flow cytometry may be predictive of the increases in aerobic capacity and endothelial function.

Keywords: Exercise training- Endothelial progenitor cells- endothelial microparticles- coronary artery disease

INTRODUCTION

Exercise training is recognized as an important preventive and therapeutic strategy in cardiovascular disease, in part through its beneficial effects on aerobic capacity and endothelial function.(19) The strong and independent prognostic role of reduced physical fitness and endothelial dysfunction has been demonstrated in healthy populations, as well as in patients with underlying coronary artery disease (CAD) and heart failure. Small single center studies show that peak oxygen uptake (peak VO_2) increases to a larger extent with aerobic interval training (AIT) at higher intensity compared to moderate intensity continuous training in patients with metabolic syndrome, after acute coronary syndrome, following coronary artery bypass surgery and in patients with ischemic heart failure.(18, 20, 31, 32) Interestingly, in the latter patient group, as well as in patients with metabolic syndrome, AIT also led to a significantly larger improvement in peripheral endothelial function.(25, 32) The reason for the superior effect of AIT on endothelial function is not completely understood. It is, however, conceivable that differences in vascular shear stress patterns between exercise protocols induce different molecular and cellular responses.

Indeed, the enhanced production of nitric oxide (NO) in response to shear stress plays a critical role in the beneficial effects of exercise training on endothelial function. In addition, the anti-inflammatory, free radical reducing and permeability decreasing properties of exercise may all contribute to improvement of endothelial function.(10)

It has also been suggested that endothelial progenitor cells (EPC) could add to these favorable changes.(16) EPC are a rather small population of cells that can be mobilized from the bone marrow into the peripheral blood by various stimuli, such as ischemia and chemokines. They participate in the repair of endothelial damage, thereby possibly influencing endothelial function.(16) EPC function is regulated by angiogenic T-cells that express the platelet endothelial cell adhesion molecule (CD31) and the receptor for stromal-

derived factor 1 (CD184).(14) Thus far, because of small-scale, single-center trials, heterogeneity in patient populations and inconsistency of study results, it was not possible to draw firm conclusions on a role for EPC and angiogenic T-cells as mediators of the training-induced improvement of endothelial function in CAD.(8)

The main objective of the Study on Aerobic INTerval EXercise training in CAD (SAINTEX-CAD) study was to investigate whether a 12-week program of AIT yields a larger gain in peak VO_2 and endothelial function compared to a similar training program of aerobic continuous training (ACT).(6) The results of this large randomized multicenter study involving 200 CAD patients demonstrated that AIT and ACT are equal in improving peak VO_2 and peripheral endothelial function.(5) The purpose of this substudy from SAINTEX-CAD is to investigate if aerobic exercise training (AIT versus ACT) can mobilize EPC and other related cellular blood markers of endothelial integrity, and to examine the relationship between these blood markers and the improvement of endothelial function.

METHODS

Patients and study design

A detailed description of the rationale and design of the SAINTEX-CAD study has been published previously.(6) Briefly, 200 stable patients with cardiovascular disease were enrolled at the Cardiac Rehabilitation centers of the University Hospitals of Antwerp (Center 1, n=100) and Leuven (Center 2, n=100), Belgium, between October 2011 and April 2013. The main study inclusion criteria were: 1) angiographically documented CAD or previous acute myocardial infarction (AMI), 2) left ventricular ejection fraction (LVEF) > 40%, 3) optimal medical treatment, 4) stable with regard to symptoms and medication for at least 4 weeks and 5) included between 4 and 12 weeks following AMI, elective percutaneous coronary Intervention (PCI) or coronary artery bypass grafting (CABG). Patients were

randomized to either aerobic interval training (AIT) or aerobic continuous training (ACT) on a 1:1 base by an online protocol at Center 1. At baseline and after 3 months, patients underwent cardiopulmonary exercise testing, vascular function assessment and blood sampling. The Laboratory of Cellular and Molecular Cardiology of Center 1 served as the central core laboratory responsible for the EPC and angiogenic T-cell analyses of both centers.

The study complies with the Declaration of Helsinki, was approved by the local ethics committees and written informed consent was obtained from each participant.

Exercise training

Thirty-six supervised exercise sessions were implemented at a rate of 3 sessions a week during 12 weeks. Patients exercised on a bicycle; exercise load was adjusted in order to comply with the target heart rate (HR) throughout the 12-week training period. Patients randomized to the AIT group cycled during 38 min in four 4-min intervals at 90–95% of peak HR (Figure 1). Each interval was separated by 3-min active pauses, cycling at 50–70% of peak HR. The session started with 10 min warm up and ended with a 3 min cool-down. Patients in the ACT group cycled continuously at an intensity of at least 70-75% of peak HR during 37 min. The session started with 5 min warm-up and ended with a 5 min cool-down.

Clinical assessments

Cardiopulmonary exercise testing

Cardiopulmonary exercise testing (CPET) was performed at baseline and after 12 weeks using an individualized cycle ergometer ramp protocol (20 Watt +20 Watt/min or 10 Watt + 10 Watt/min). Breath-by-breath gas exchange measurements allowed on-line determination of ventilation (VE), oxygen uptake (VO_2) and carbon dioxide production (VCO_2) every 10 s. Peak VO_2 was determined as the mean value of three measures of VO_2 during the final 30 s

of exercise. The anaerobic threshold (AT) and the respiratory exchange ratio (RER) were recorded.

Endothelium-dependent vasodilation

All analyses were performed in the morning, in fasting conditions and in a quiet temperature-controlled room (21-24°C) by a trained operator that was blinded for the study intervention. Subjects refrained from exercise, food and caffeine at least 8 hours before the measurements. Blood pressure was obtained after 10 minutes of rest with an automated blood pressure monitor. Endothelial function was assessed by flow-mediated dilation (FMD) of the brachial artery using ultrasound (Center 1, AU5 Ultrasound System, Esaote; Center 2, GE Healthcare, Vivid 7), according to the International Brachial Artery Reactivity Task Force guidelines.(7) A high-resolution linear-array vascular probe was used (Center 1, 10 MHz; Center 2, 5-13 MHz). Patients were positioned supine with the right arm resting on an arm support; the brachial artery was imaged above the antecubital fossa in the longitudinal plane. After recording of the baseline diameter for at least 1 minute of stable distension waveforms, a blood pressure cuff at the forearm was inflated to at least 200 mmHg or 60 mmHg higher than the resting systolic blood pressure. After cuff deflation, images were recorded for 3 minutes. Images were analyzed using edge-detection software FMD-i by Flomedi (Flomedi, Brussels, Belgium). FMD was expressed as the change in post-stimulus diameter as a percentage of the baseline diameter. Analyses were blinded in both study centers.

Flowcytometric quantification of cellular markers of endothelial integrity

Quantification of EPC

EPC were defined as CD34+KDR+CD45dim cells.(23) Fixated whole blood (TransFix, Caltag Medsystems, Buckingham, UK) was processed 2 to 3 days after sampling.(13) Red blood cells

were lysed using ammonium chloride solution (NH_4Cl). After pre-treatment with Fc receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), samples were stained with the following antibodies: CD34-PE-Cy7 (BD Pharmingen, Erembodegem, Belgium), KDR-APC (R&D Systems, Minnesota, USA), CD45-APC-H7 (BD Pharmingen). Negative controls included fluorescence-minus-one samples and unstained samples. The nucleic acid dye SYTO 13 (Life Technologies, Gent, Belgium) allowed exclusion of non-nucleated cells and cellular debris. At least 1 million total events were recorded on a FACSCanto II flow cytometer (Becton Dickinson, New Jersey, USA). Numbers of EPC were analyzed using FACSDiva software (Becton Dickinson, version 6.1.2) and expressed as cells per million CD45+ mononuclear cells with low forward (FSC) and side scatter (SSC). Briefly, after exclusion of cellular aggregates (FSC area versus height plot) and debris (SYTO 13 negative), a primary gate was set on the mononuclear cells. Next, a second gate was set on a CD45 versus SSC dot plot to contain all $CD45^{dim}$ events, as previously described.(23) CD34+ and KDR+ events were analyzed in this population.

Quantification of angiogenic T-cells

Angiogenic T-cells were defined as CD31+CD3+CD184+ cells.(14) After red cell lysis and Fc receptor blocking at day 2 or 3, fixated whole blood was stained with CD31-FITC, CD3-PerCP, and CD184-APC antibodies (all from BD Biosciences). Unstained samples and fluorescence-minus-one samples for CD31 and CD184 were used as controls. At least 500.000 total events were analyzed using FACSDiva software and expressed as cells per million mononuclear cells. Doublets and aggregates were excluded by selecting singlet cells on a FSC area versus FSC height plot.

Quantification of EMP

EMP were defined as CD31+CD42b- particles smaller than 1 µm (Fluoresbrite YG 1µm calibration size beads, Polysciences, Eppelheim, Germany). EMP enumeration was performed only on samples collected at Center 1 (n= 90). For this purpose, platelet poor plasma (PPP) was produced immediately after blood sampling by double centrifugation at 1550x g. Antibodies used were CD31-PE and CD42b-FITC (both from BD Biosciences). Samples were analyzed as we previously described, enabling the evaluation of circulating EMP per µl PPP. (28)

Biochemical assays

Complete blood count was measured on Advia Haematology Analyzer (ADVIA 2120, Siemens Healthcare Diagnostics). Levels of creatinine and high sensitivity C-reactive protein (hs-CRP) were measured using routine laboratory techniques (Dimension Vista 1500 System, Siemens). Estimated glomerular filtration (eGFR) was calculated using the MDRD formula.

Statistical analysis

Continuous data are expressed as mean ± standard deviation (SD). Skewed distributed data (one-sample Kolmogorov Smirnov test) are presented as median (range). Baseline comparisons were performed using independent sample T test or X^2 test where appropriate. Differences over time between groups (=interaction) were analyzed by univariable two-way repeated measures analysis of variance (ANCOVA) with age and pathology as covariates. Whereas no centre-effect was found for peak VO2, FMD values were significantly higher in center 2. Therefore, ANCOVA for FMD included age, pathology and center as covariates. Percentual changes of FMD were skewed and therefore expressed as median (range). Pearson or Spearman correlation coefficients were used for correlations. A stepwise multiple linear regression analysis was used to assess independent determinants of peak VO_2 changes with adjustment for significant determinants on correlation analysis. All tests

were two-sided, and a p-value of 0.05 was considered statistically significant. All analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Demographic and clinical characteristics of the patients are shown in Table 1. Age and pathology differed significantly, with younger age, more post-AMI and less post-PCI patients in the AIT group, while other baseline values and pharmacological treatment were similar between AIT and ACT.

Baseline levels of endothelial integrity markers

Numbers of circulating EPC, angiogenic T-cells and EMP were not associated with the presence of cardiovascular risk factors like hypertension, diabetes and smoking (all $p>0.05$). In addition, numbers did not correlate with age, hs-CRP or eGFR (all $p>0.05$). There were no differences in EPC, angiogenic T-cells and EMP numbers between post-AMI, post-PCI or post-CABG patients (all $p>0.05$).

Changes in clinical parameters post-training

As previously reported, mean training intensity was 88% of peak HR in the AIT group and 80% of peak HR in the ACT group.(5) Peak VO_2 was significantly higher in both groups after 12 weeks (AIT 22.7±17.6% versus ACT 20.3±15.3%; p-time<0.001, Table 2). In addition, FMD improved significantly in both groups (AIT median 34.1% (range -69.8 to 646%) and ACT median 7.14% (range -66.7% to 503%); p-time<0.001). The improvement in peak VO_2 was correlated to improvement in FMD (pearson $r = 0.17$, $p = 0.035$). Improvements in both outcomes were comparable for both training interventions.

Changes in numbers of EPC, angiogenic T-cells and EMP post-training

After 12 weeks of AIT or ACT, numbers of EPC, angiogenic T-cells and EMP in peripheral blood were comparable to baseline levels (all $p>0.05$, Table 2). This was observed for both AIT and ACT groups. Results were comparable between the three different etiologies: we observed no significant change in the number of EPC, angiogenic T-cells or EMP, neither in the post-AMI group, nor in the post-PCI or post-CABG group (all $p>0.05$). Various clinical (age, BMI and systolic blood pressure) and biochemical variables (hs-CRP, eGFR, LDL cholesterol and leukocytes) were evaluated for their possible interference effects on EPC, angiogenic T-cells and EMP. None of these variables were related to changes in markers of endothelial integrity (all $p>0.05$).

Cellular markers as predictors of the training-induced response

At baseline, no correlations were observed between cellular markers and endothelial function or aerobic capacity. However, baseline numbers of EMP were related to the magnitude of the change in peak VO_2 (spearman rho = -0.245, $p = 0.027$) and the change in FMD (spearman rho = -0.374, $p = 0.001$) following exercise training. For this analysis, the total cohort was pooled (AIT and ACT) since an interaction term was absent and EMP numbers were logarithmically transformed. These relations were maintained in multivariate regression analysis of logarithmically transformed EMP numbers and after correction for baseline peak VO_2 and age, variables that were related to the change in peak VO_2 in univariate analysis (beta -0.263, $p = 0.01$). Baseline EPC or angiogenic T-cells did not correlate with changes in FMD or peak VO_2.

DISCUSSION

To our knowledge, the current substudy of the SAINTEX-CAD trial is the largest randomized, multicenter study evaluating the effect of exercise training on cellular blood markers of endothelial integrity in stable CAD patients. Despite a significant improvement in peripheral endothelial function, we found no meaningful changes in the numbers of circulating EPC, angiogenic T-cells and EMP after 12 weeks of AIT or ACT. EMP counts at baseline, however, were related to the improvements in peak VO_2 and FMD at completion of the training program.

Blood related markers of endothelial damage and repair

Endothelial dysfunction precedes overt atherosclerosis by many years and is an independent prognostic marker of cardiovascular events.(22) Disruption of endothelial homeostasis results from imbalances in the production of nitric oxide and reactive oxygen species, local and systemic low-grade inflammation and loss of endothelial cells by apoptosis.(4) The injury of the vessel wall leads to the recruitment of circulating EPC and angiogenic T-cells to the site of endothelial disruption. It is well known from human and animal studies that EPC and angiogenic T-cells, which stimulate EPC function, actively participate in the repair of damaged endothelium.(29, 30) Moreover, endothelial repair seems to improve with their increased numbers in the circulation, reflecting a higher regenerative capacity.

High levels of EMP affect the endothelial cell layer lining in a negative manner, in contrast to EPC and angiogenic T-cells. EMP are shed from the plasma membrane of endothelial cells upon their activation, apoptosis or injury.(12) These small particles may contribute to worsening of endothelium injury by impairing the endothelium-dependent vasodilation and modulating inflammation via leukocyte activation and trans-endothelial migration.(9)

Impact of exercise training on endothelial function and endothelial repair

This is the first study to examine the influence of exercise training on circulating levels of EMP and angiogenic T-cells in patients with CAD. Data regarding the impact of physical training on the number of EPC, however, are conflicting. One recent study reported an increase in CD34+KDR+CD45dim EPC at the completion of a 4 week aerobic exercise training program in 61% of stable patients with a previous AMI (n=112).(3) In that study, patients in the lowest tertile of baseline hs-CRP were most likely of obtaining an increase in EPC. Variations in peak VO_2 were correlated with variations in EPC, and patients without an increase in peak VO_2 (n = 26) demonstrated a lower improvement in EPC number as compared to patients with an increase in peak VO_2. Likewise, Ikeda et al. described that a 3 month walking program of > 4 hours walking per week, led to a gain in aerobic capacity and CD34+CD133+ EPC number in patients with recent AMI (n=23).(15) Moreover, Steiner et al. provided evidence supporting a role for increased CD34+KDR+CD133+ EPC in the augmentation of endothelial function during exercise in CAD patients (n=20).(24) Three months of aerobic exercise training resulted into a higher EPC level, which was positively correlated with the change in FMD. Paul et al. also reported a rise in CD133+KDR+ EPC in 35 out of 46 CAD patients after three months of aerobic exercise training.(21) Brachial artery FMD, however, was not improved and did not correlate with the number of EPC. In addition, the reduction in plasma hs-CRP was modest and did not reach statistical significance at program completion. Finally, Luk et al. (n = 32) and Hansen et al. (n = 47) did not find a significant increase in CD34+KDR+ EPC in patients with CAD after 8 and 6 weeks of aerobic exercise respectively.(11, 17) Although we must acknowledge the considerable variation in EPC phenotypes, our results are more consistent with these latter studies, indicating that EPC, angiogenic T-cells and EMP are not critically involved in mediating the training-induced improvement of endothelial function in CAD patients.

Responders to exercise training

To date, it is widely recognized that the individual response to exercise training in terms of aerobic capacity is highly variable among patients. Aerobic capacity is one of the strongest prognosticators in cardiovascular disease, but 20% of patients have a low or absent response in peak VO_2 to training.(1) The mechanisms driving this variability are not well understood nor do we have good predictors of the response to exercise therapy. Heritability accounts for 45–50% of the anticipated effect of exercise training.(2) In the present study, EMP count at baseline was an important within-patient predictor of the change in peak VO_2 and peripheral endothelial function at completion of a 3-month training program. Although additional studies are needed to confirm its predictive value, EMP flow cytometry may offer guidance to clinicians and physiotherapists in order to tailor exercise protocols to the need of individual patients and thereby maximize the beneficial effects.

Limitations

The definition of EPC is still a matter of debate. In our study, EPC enumeration was performed according to the recommendations of Van Craenenbroeck et al.(27) The phenotypic profile of EMP may change according to the type of vascular injury (activation or apoptosis). It is therefore unlikely that our set of markers efficiently labeled the entire EMP population. A final limitation is that we could not include a functional analysis of circulating angiogenic cells in this study design. Circulating angiogenic cells contribute to endothelial repair in a paracrine fashion. In previous work, we found that exercise induces favorable effects on the functional capacity of these cells in chronic heart failure patients.(26)

Conclusions

Our results demonstrate that the improvement of endothelial function in response to exercise training in stable CAD patients is not mediated by an increased release of EPC and

angiogenic T-cells nor to a diminished shedding of EMP into the peripheral circulation. EMP counts at baseline, however, may be predictive of the extent of increase in aerobic capacity and endothelial function at completion of the training program.

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FIGURE LEGENDS

Figure 1. Schematic illustration of both training programs.

TABLES

Table 1. Demographic and clinical characteristics at baseline

	AIT n=100	ACT n=100	P-value
Age (yrs)	57.0 ± 8.8	59.9 ± 9.2	0.023
Gender (M/F)	91/9	89/11	NS
LVEF (%)	57.1 ± 8.5	56.8 ± 7.7	NS
Cardiovascular risk			
Body mass index (kg/m^2)	28.0 ± 4.4	28.5 ± 4.3	NS
Diabetes (%)	20	18	NS
Hypertension (%)	58	46	NS
Smoking (%)	73	74	NS
Reason for referral			
CABG (%)	26	34	NS
Elective PCI (%)	7	18	0.019
AMI (%)	67	48	0.007
Laboratory measurements			
Hemoglobin (g/dl)	14.3 ± 1.3	14.2 ± 1.4	NS
Leukocytes (cells/µl)	6698 ± 1575	1042 ± 2094	NS
eGFR (ml/min/1.73 m^2)	78.9 ± 12.9	77.5 ± 12.6	NS
hs-CRP (mg/l)	4.71 ± 9.4	3.45 ± 7.2	NS
Exercise Capacity			
Peak VO_2 (ml/kg/min)	23.3 ± 5.8	22.2 ± 5.6	NS
% VO_2predicted	82.8 ± 22.6	83.3 ± 22.7	NS
Maximal workload (Watts)	152 ± 39	144 ± 41	NS
Medication			
Statin (%)	97	99	NS
Aspirin (%)	93	95	NS
Beta-blocker (%)	84	83	NS
ACE-inhibitor/ARB (%)	77	72	NS

Values are mean (±SD) or percentage (%). NS: not significant.

LVEF= left ventricular ejection fraction; CABG= coronary artery bypass graft; PCI= percutaneous coronary intervention; AMI= acute myocardial infarction; eGFR= estimated glomerular filtration rate; hs-CRP= high sensitivity C-reactive protein; VO_2= oxygen uptake; ACE= angiotensin converting enzyme; ARB= angiotensin II receptor blocker

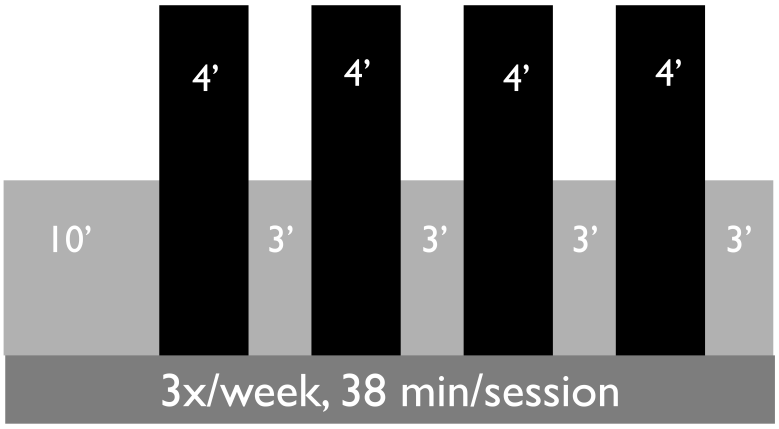
Table 2. Endothelial function and endothelial integrity markers following AIT or ACT

	AIT		ACT			
	0 weeks	12 weeks	0 weeks	12 weeks	F value time	F value Interaction
Peak VO_2 (ml/kg/min)	23.5 ± 5.7	28.6 ±6.9	22.4 ±5.6	26.8 ±6.7	28.18 *	0.16 (NS)
FMD (%)	5.26 ±3.02	6.47 ±2.79	5.61 ±2.36	6.68 ±3.04	7.28 *	0.06 (NS)
EPC (/10^6 MNC)	8.2 (0-51)	7.4 (0-53)	9.5 (0-37)	10.6 (0-106)	0.13 (NS)	1.8 (NS)
Angiogenic T-cells (/10^6 MNC)	1901 (186-20494)	1950 (117-26247)	2744 (132-24000)	4765 (164-31392)	0.95 (NS)	1.23 (NS)
EMP (/µl)	129 (47-756)	192 (47-755)	227 (80-715)	260 (60-922)	0.0 (NS)	0.8 (NS)

Values are mean (±SD) or median (range) * p< 0.001, NS: not significant. EPC, angiogenic T-cells and EMP data were log transformed before analysis. ANCOVA with age and pathology as covariates was performed to test time and interaction effects.

AIT= aerobic interval training; ACT= aerobic continuous training; VO2= oxygen uptake; FMD= flow mediated dilation; EPC= endothelial progenitor cells; EMP= endothelial microparticles; MNC= mononuclear cells.

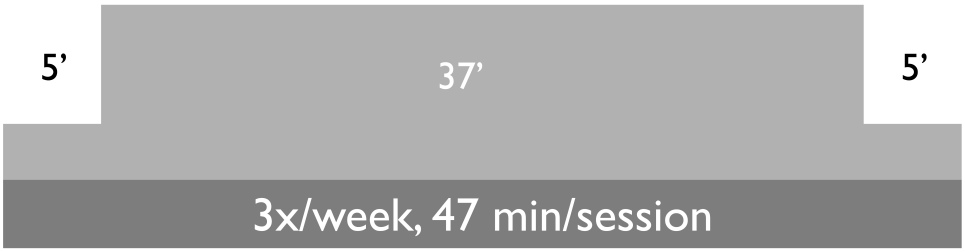
AIT



90-95% peak HR

50-70% peak HR

ACT



70-75% peak HR

AIT= Aerobic interval training; ACT= Aerobic continuous training; HR= heart rate